Tools for Documenting the Establishment of Microbial Biocontrol Agents

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Microbial Biocontrol of Arthropods, Weeds, and Plant Pathogens: Risks, Benefits and Challenges
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Biological Control of Plant Diseases

Approaches

- Indigenous organisms: Natural microbially-based defense

Suppressive Soils

pathogen does not establish or persist

- **establishes but causes little or no damage**
- **establishes, causes disease but then declines in severity**
- **pathogen may persist in the soil** (Baker and Cook, 1974)

- Introduced microbial agents

Many organisms have been studied for biocontrol activity but research has focused on *Pseudomonas*, *Bacillus* & *Trichoderma*.

Characteristics of Biocontrol Agents

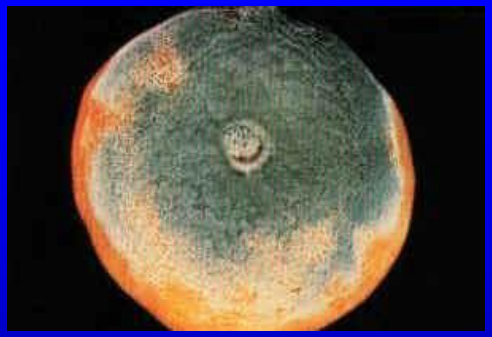
- ***Bacillus***
  - ♦ bacterium of choice for commercial BCAs
  - ♦ easily mass produced
  - ♦ easily formulated; forms a dormant, resistant spore
- ***Trichoderma***
  - ♦ fungus of choice for commercial BCAs
  - ♦ easily mass produced
  - ♦ sporulates profusely, easily formulated
  - ♦ resistant to natural and synthetic chemicals
- ***Pseudomonas***
  - ♦ organism of choice for fundamental studies of biocontrol
  - ♦ easily mass produced, but harder to formulate; no spores
  - ♦ few commercial BCAs

Tracking Biocontrol Agents

Foliar diseases

Soilborne diseases

Postharvest diseases



Soil and rhizosphere: most complex environments in which BCAs must establish and function

Tracking BCAs: complicated by the microbial milieu in the rhizosphere

Characteristics of Root Colonization by Bacterial BCAs

- BCAs applied to seeds or planting material spread throughout a root system but cells are not uniformly distributed along roots (Bahme & Schroth, 1987; Bull et al., 1991; Loper et al., 1984).
- Densities of BCAs are greatest after planting, decline throughout the growing season or decline & stabilize; populations do not exceed $5x10^8$ CFU g^{-1} root + rhizosphere soil (Bakker et al., 1986; Kluepfel, 1993; Mahaffee et al., 1997; Steddom et al., 2002; Weller, 1983).
- The proportion of the heterotrophic bacterial population comprised by a BCA is greatest soon after planting and decreases throughout the growing season (Halverson et al., 1993; Juhnke et al., 1989; Weller, 1983).

Tools for Tracking BCAs

Seeking Sensitivity and Specificity

- **Direct Microscopy:** (lacks specificity)
  - ♦ **Traditional SEM:** extensive sample preparation
    Sugar beet seed colonization *B. subtilis* & *P. putida* (Fukui et al., 1994)
    - Distribution of *P. fluorescens* on pea roots (Dandurand et al., 1997)
  - ♦ **Environmental Scanning Electron Microscopy**
    - Water remains liquid and specimens are hydrated; no preparation
- **Molecular Stains:** staining and tagging systems & advanced fluorescence microscopy; detection of single cells in complex environments; greater sensitivity
  - ♦ **General Cell Stains**
    - Epifluorescence microscopy & DNA staining: acridine orange (DeLeo et al., 1997); SYBR Green II (Weinbauer et al., 1998)
    - Fluorescent Brighteners: staining of fungi (Thrane et al., 1999; Eickhorst & Tippkötter, 2008; Harris et al., 2002)

Sørensen et al., 2009

Tools for Tracking BCAs

Seeking Sensitivity and Specificity

- **Specific Cell Stains**
  - **Confocal Laser Scanning Microscopy (CLSM) with Strain-Specific Fluorescent Antibody Staining (enhanced sensitivity and specificity)**
    - High resolution 3-D images of structure and composition of microbial communities
    - Early studies: *A. brasilense* and *P. fluorescens* colonizing wheat/barley roots (Schloter et al., 1993; Hansen et al., 1997; Kirchhof et al., 1997)
  - **CLSM with Fluorescence In Situ Hybridization (FISH) (targeting rRNA & mRNA)**
    - Numerous studies of root colonization (Assmus et al., 1995, 1997; Watt et al., 2006; Santaella et al., 2008)
    - FISH showed different colonization patterns for *Salmonella enterica* and *Listeria* spp. on barley roots (Kutter et al., 2005)

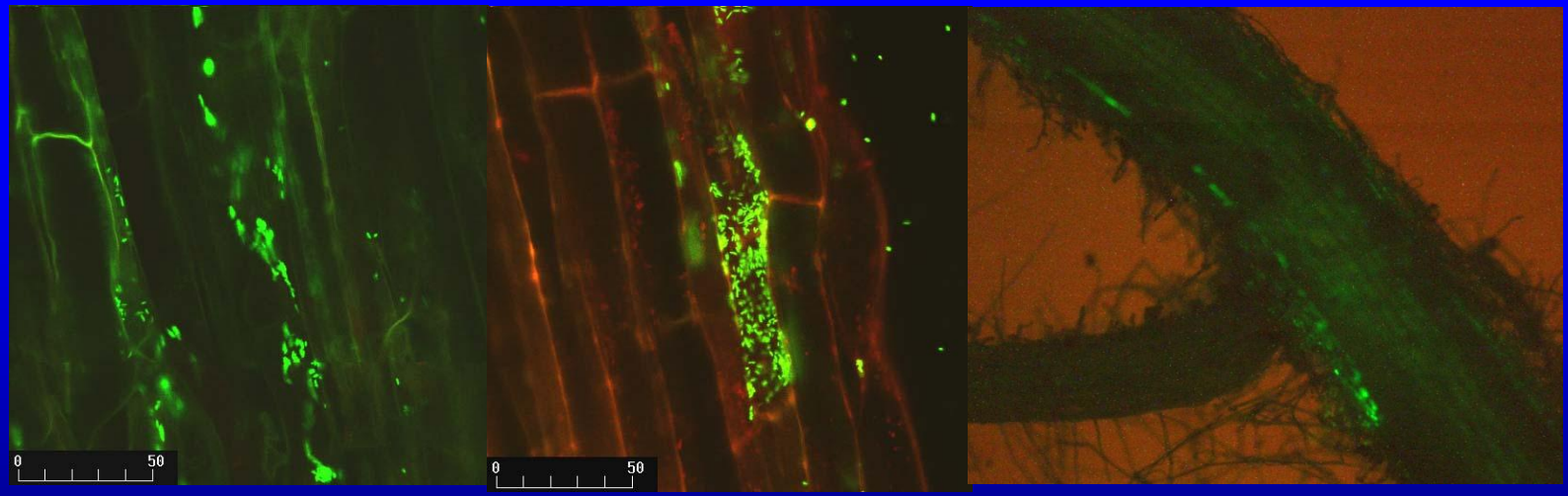
Sørensen et al., 2009

Tools for Tracking BCAs

Seeking Sensitivity and Specificity

- **Reporter Strains:** single strains carrying fluorescence marker genes
  - **Non-Specific reporter:** *lux* of *gfp* genes controlled by a constitutive promoter (bioluminescence/ green fluorescent protein)

    - CLSM used to track GFP mutants of *P. chlororaphis* on barley seed (Tombolini et al., 1999); *P. fluorescens* on roots of barley (Normander et al.,1999), tomato (Gotz et al., 2006), avocado (Pliego et al., 2008), olive (Prieto & Mercado-Blanco, 2008), tomato (Chin-A-Woeng et al., 1997; Bloemberg et al., 2000)



CFLM CFLM EFM

Wheat roots colonized by *gfp*-tagged *P. fluorescens Q8r1-96* (10 days PI)

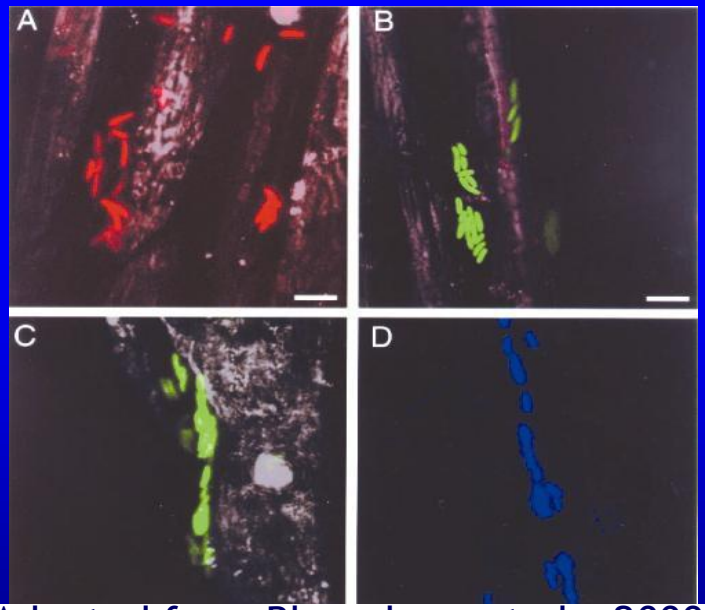
Tools for Tracking

Simultaneous imaging of *Pseudomonas fluorescens* WCS365 expressing autofluorescent proteins in the rhizosphere.

- GFP – green fluorescent protein (isolated from *Aequorea victoria*)

Derivatives of GFP

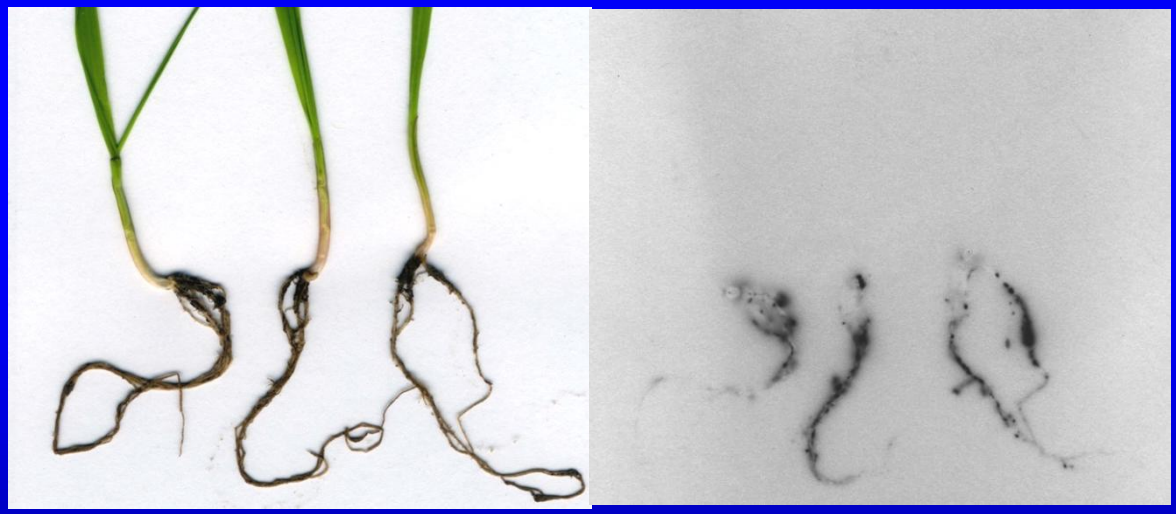
- ECFP – enhanced cyan fluorescent protein; expressing bacteria appear red (A)
- EGFP – enhanced green fluorescent protein; expressing bacteria appear green (B)
- EYFP – enhanced yellow fluorescent protein; expressing bacteria appear green (C)
- RFP – red fluorescent protein; expressing bacteria appear blue (D) (isolated from *Discosoma* spp.)



Adapted from Bloemberg et al., 2000

Tools for Tracking BCAs

- **Reporter Strains:**
  - **Semi-Specific reporter:** responds to exposure to particular conditions (high temperature; ROS etc.)
  - **Specific reporter:** responds to presence (Jager et al., 1999) or absence (Koch et al., 2001) of specific compounds or elements



T3SS genes of Q8r1-96 expressed in the wheat rhizosphere

X-ray film image of roots colonized by Q8r1-96 tagged by a mini-Tn*7* transposon with the *rspJ* promoter fused to luciferase genes (Mavrodi et al., in press)

Well-Studied Groups of BCAs: PCA- & DAPG-Producing *Pseudomonas* spp.

Phenazine-1-carboxylic acid (PCA)

P. fluorescens 2-79

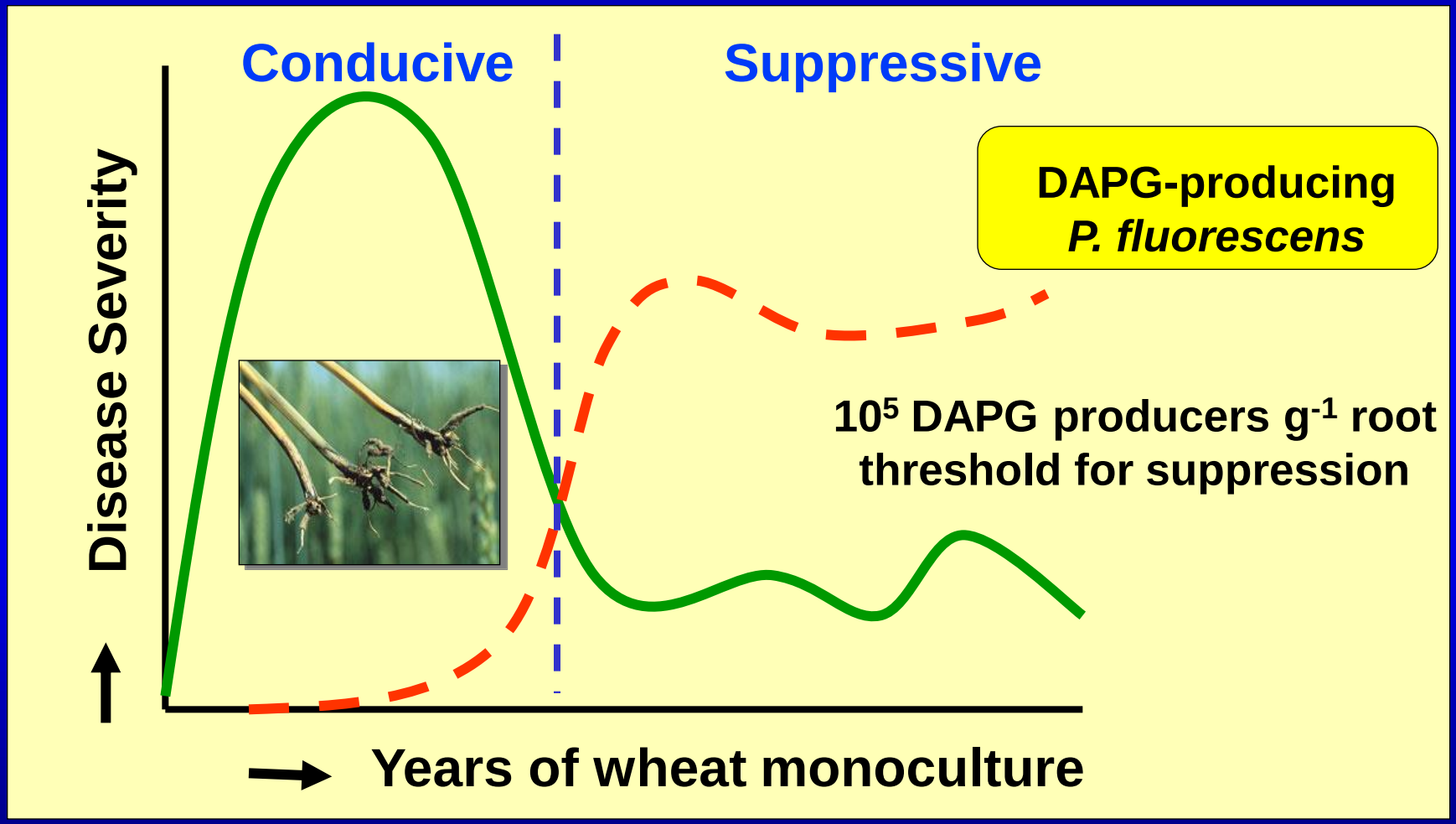
2,4-Diacetylphloroglucinol (DAPG)

P. fluorescens Q8r1-96

Take-all

Gaeumannomyces graminis* var. *tritici

Take-All Decline (TAD)



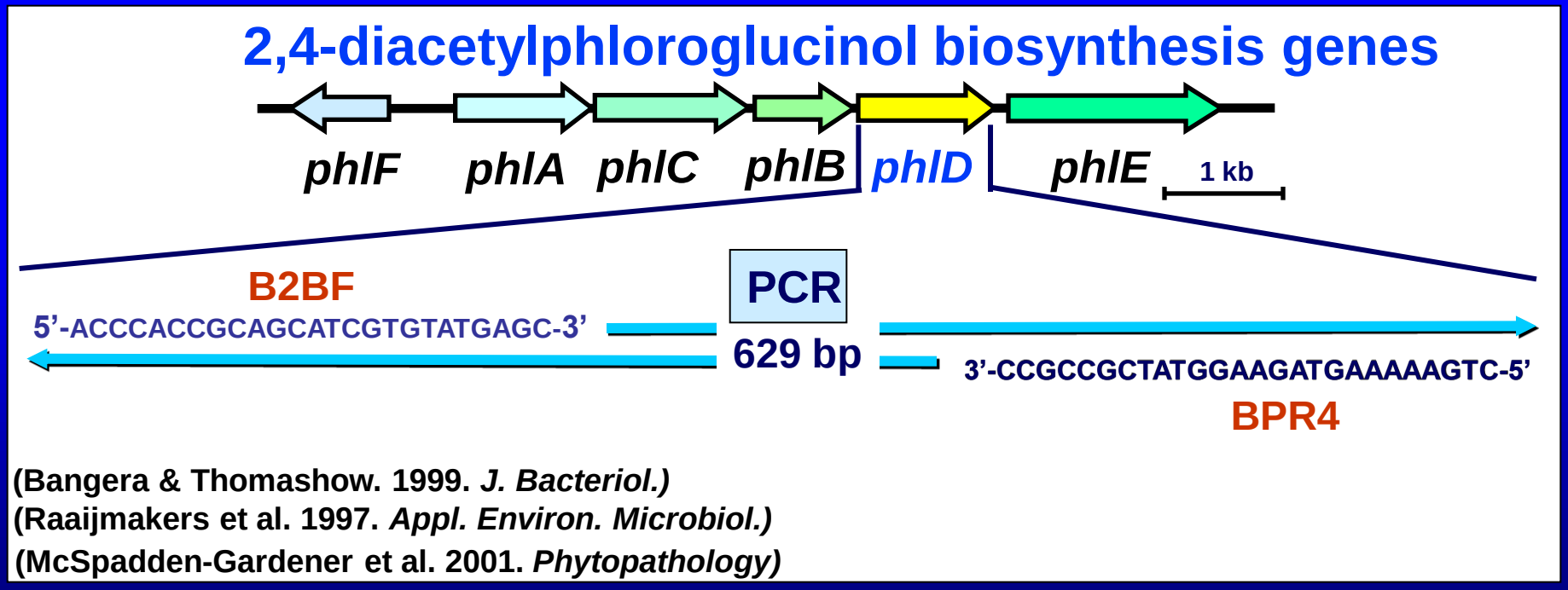
Weller et al. 2002. *Ann. Rev. Phytopathol*.

Detection of DAPG-Producing Fluorescent *Pseudomonas* spp. (*phlD*$^+$)

phlD encodes for a polyketide synthase; **genetic marker**

phlD specific probes and primers used in combination with colony hybridization and PCR to detect DAPG producers

All DAPG producers contain *phlD*



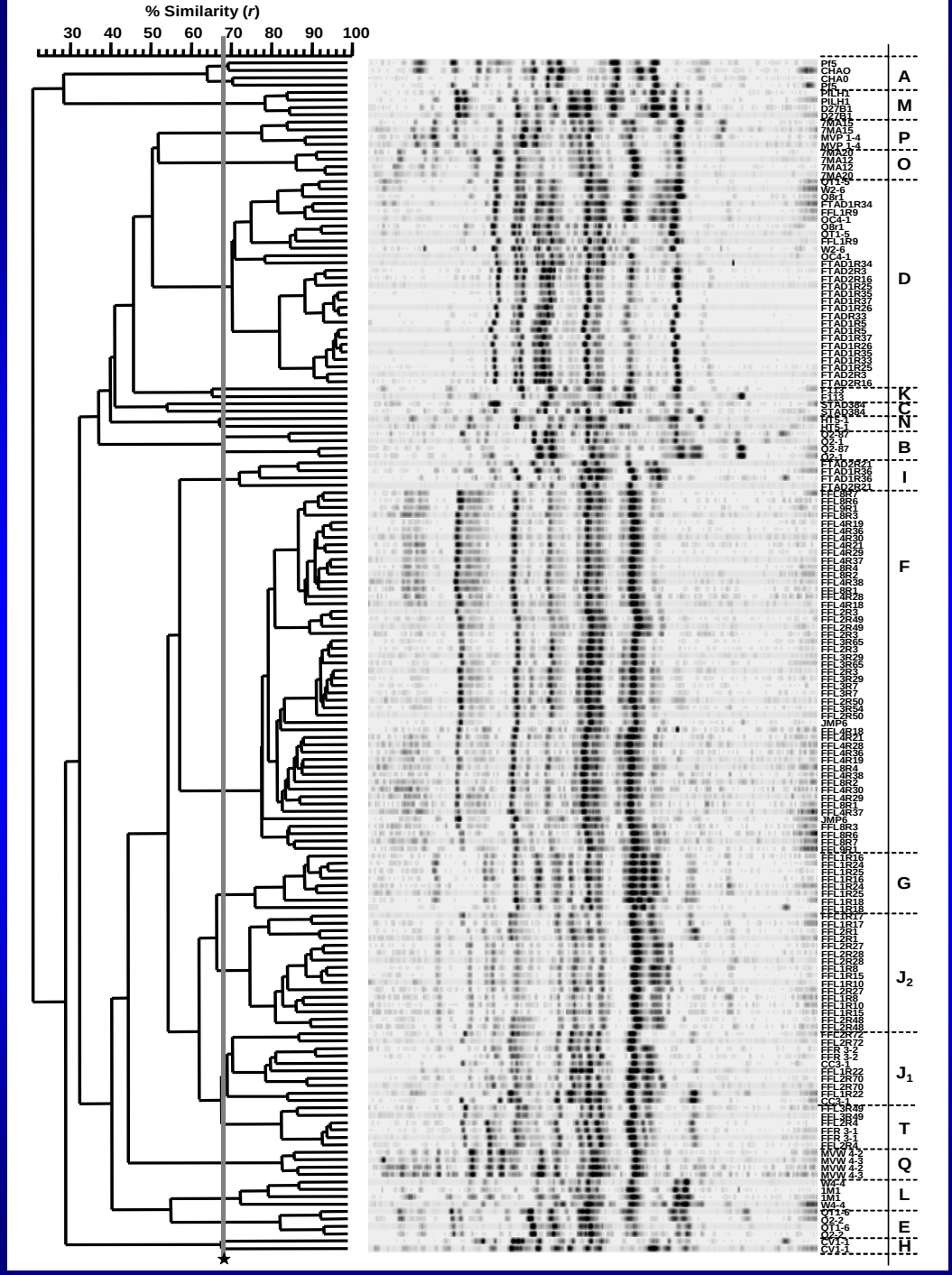
(Bangera & Thomashow. 1999. *J. Bacteriol.)*
(Raaijmakers et al. 1997. *Appl. Environ. Microbiol.)*
(McSpadden-Gardener et al. 2001. *Phytopathology)*

Diversity of DAPG Producers

- *phlD*-RFLP
- Rep-PCR (BOXA1R primer)

- 22 Genotypes
  (A-T, PfY, PfZ)

- D-genotype dominant in Washington TAD soils



Landa et al., 2005
Raaijmakers & Weller, 2001
Landa et al., 2002
McSpadden Gardener et al., 2000

Tools for Tracking BCAs

Seeking Sensitivity and Specificity

- **Culture-Based Methods**

  Selective or Semi-Selective Media

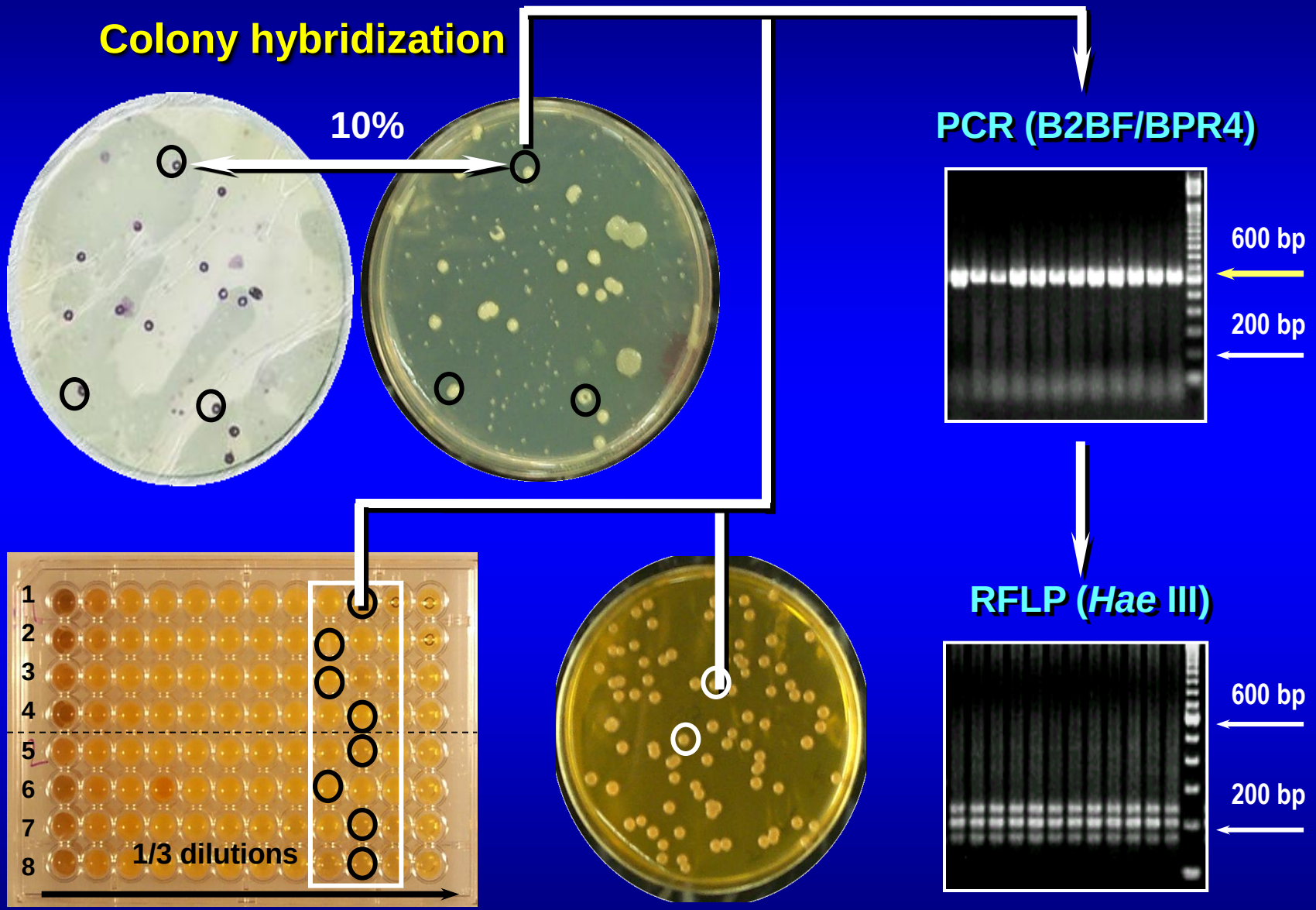
  Pseudomonas (Sands & Rovira, 1970; Simon & Ridge, 1974; Grant & Holt, 1977)

  - **Antibiotic Resistant Strains**
    - Most common approach; rifampicin resistant derivatives (Weller, 1984; Mahaffee et al., 1997)
  - **Immunofluorescent Colony Staining** (antigenic specificity)
    - Uses strain-specific antibodies; cells remain viable; highly sensitive (Mahaffee et al., 1997; Van der Wolf et al., 1995, 2000)
  - **Colony Hybridization** (gene-based specificity)
    - Bacterial colonies are replica-plated onto membranes and their DNA is hybridized with a gene-specific probe
    - Identification of DAPG-producing *P. fluorescens* (Raaijmakers et al., 1997)

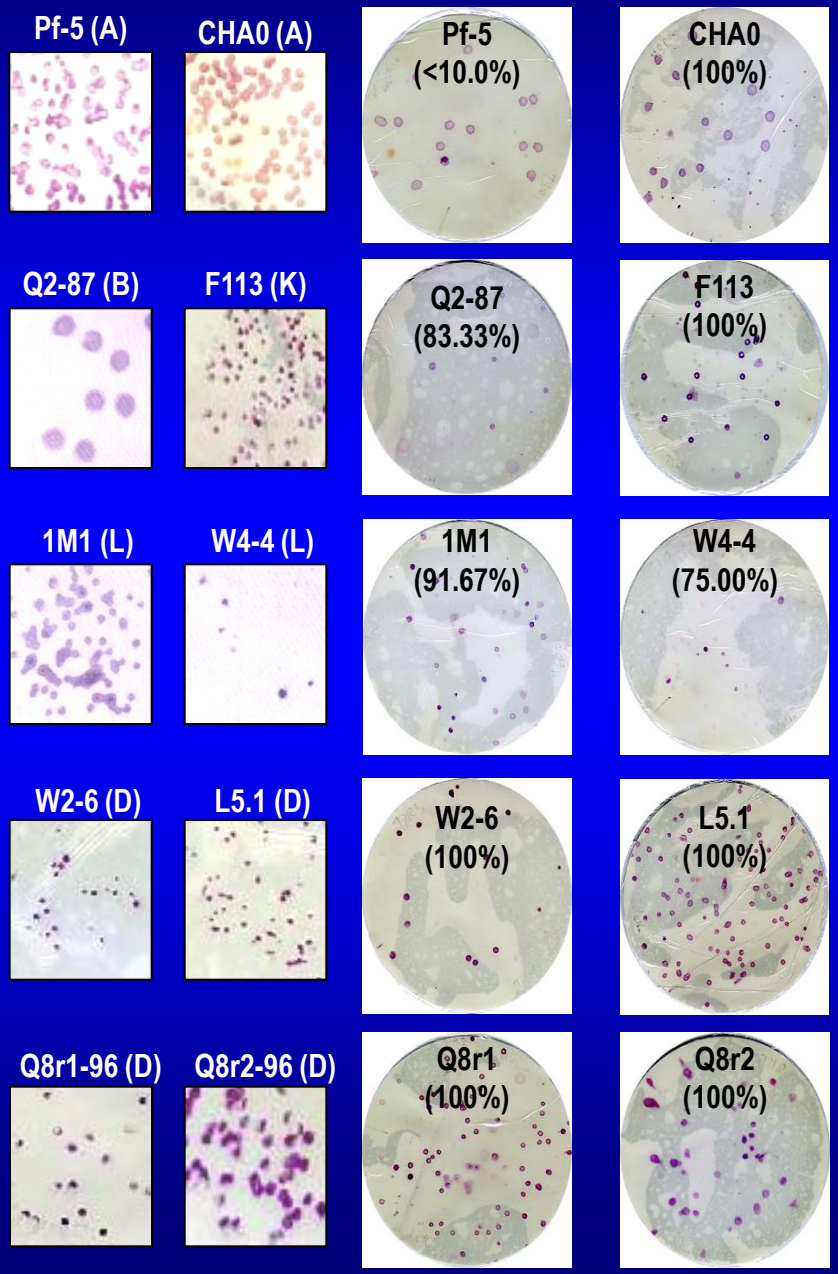
Tools for Tracking BCAs

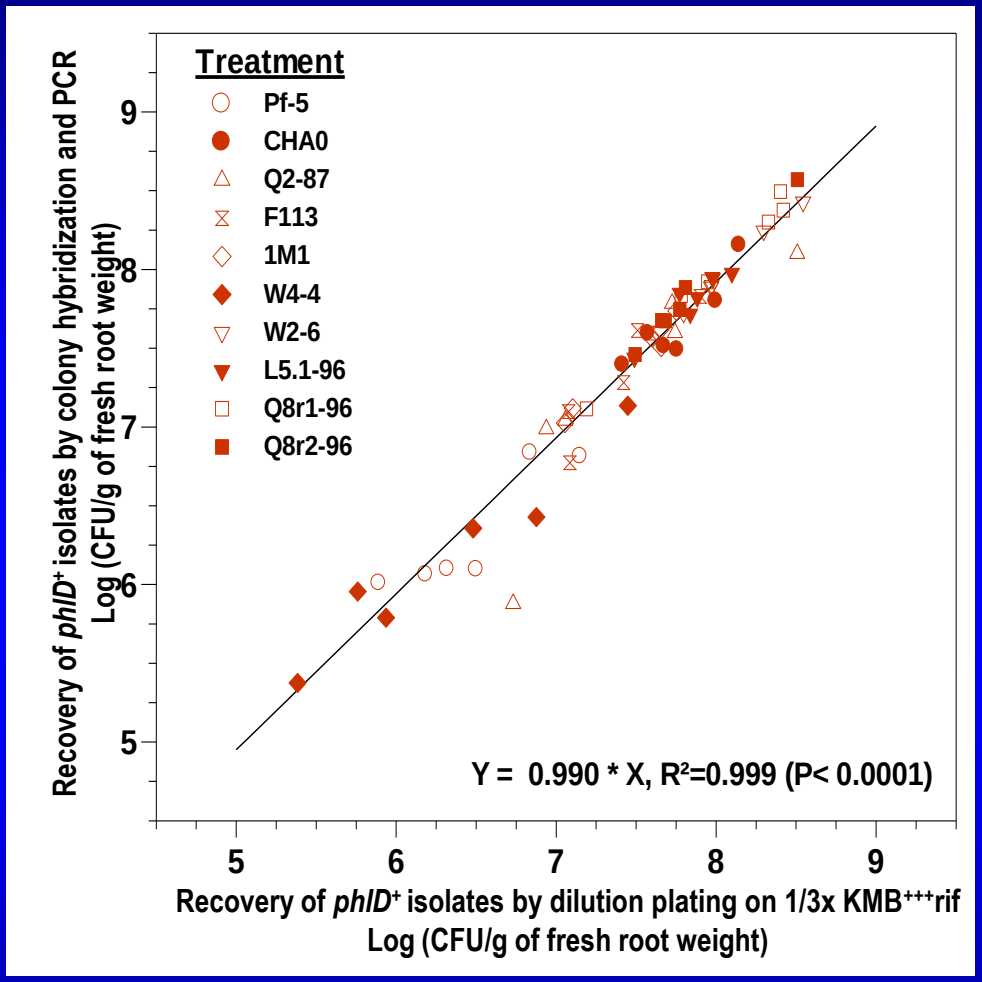
- Culture-Based Methods:
  - **PCR-Based Dilution-Endpoint Method:**
    - three fold dilution in microtiter plates with selective media followed by PCR with specific probes (McSpadden Gardener et al., 2001; Landa et al., 2002)
- Culture-Independent Quantitative PCR: (Rezzonico et al., 2003)
  - Mavrodi et al., (2007) used a real-time PCR SYBR green assay to quantify different genotypes of DAPG-producing *P. fluorescens* on the roots of wheat.

Identification of DAPG-producing *Pseudomonas* spp. from the rhizosphere



Colony Hybridization & PCR vs. Antibiotic Resistance

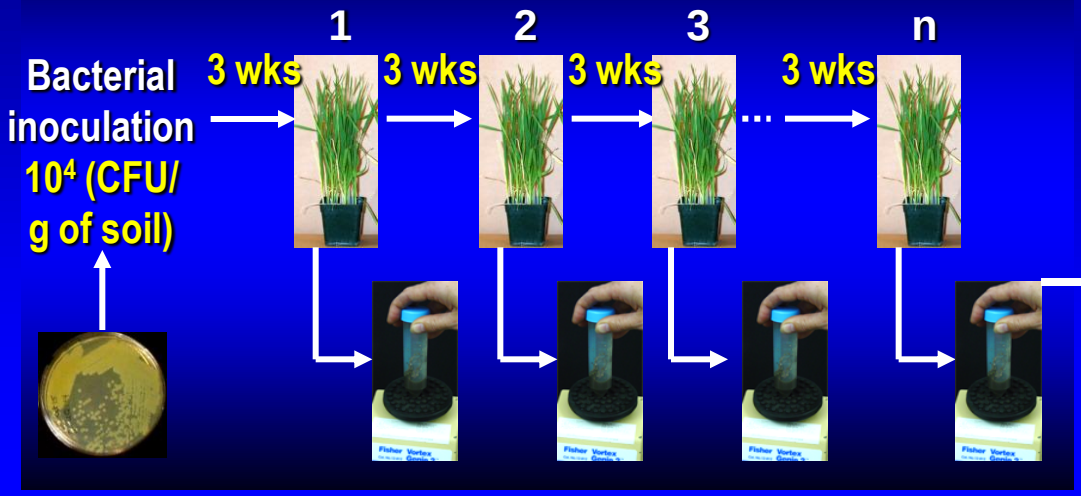




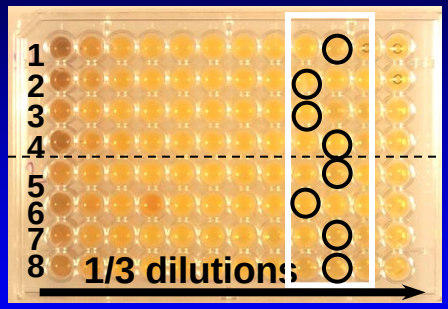
Relationship of population size of DAPG producers estimated by dilution plating onto rifampicin-containing media and by colony hybridization followed by PCR (Landa et al., 2002)

Identification of DAPG-Producing *Pseudomonas* spp. from the Rhizosphere

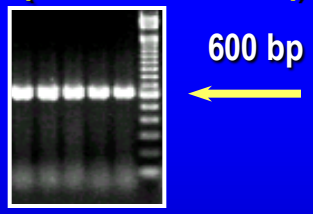
Cycling experiments



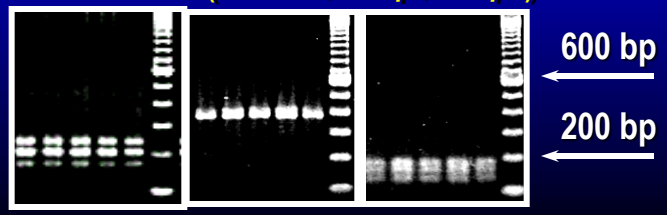
PCR-based assay



PCR (B2BF/BPR4)

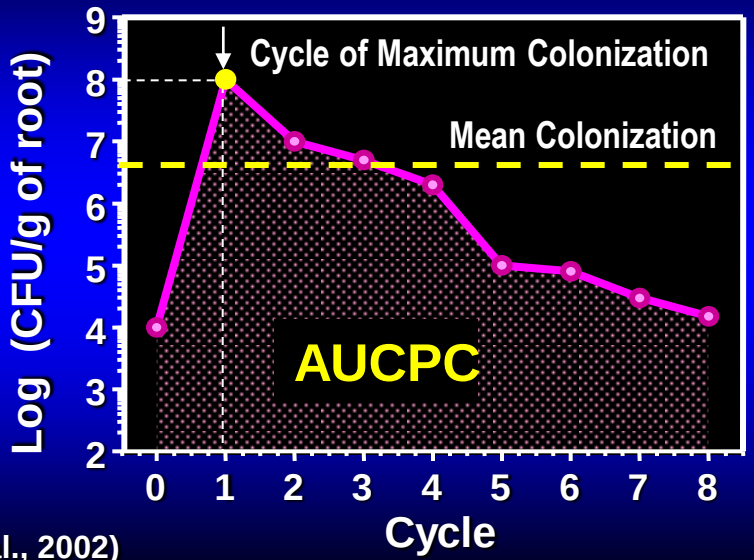


RFLPs (*Hae*III, *Taq*I, *Msp*I)



(McSpadden Gardener et al., 2001)

Comparison of treatments



(Landa et al., 2002)

Tools for Tracking BCAs

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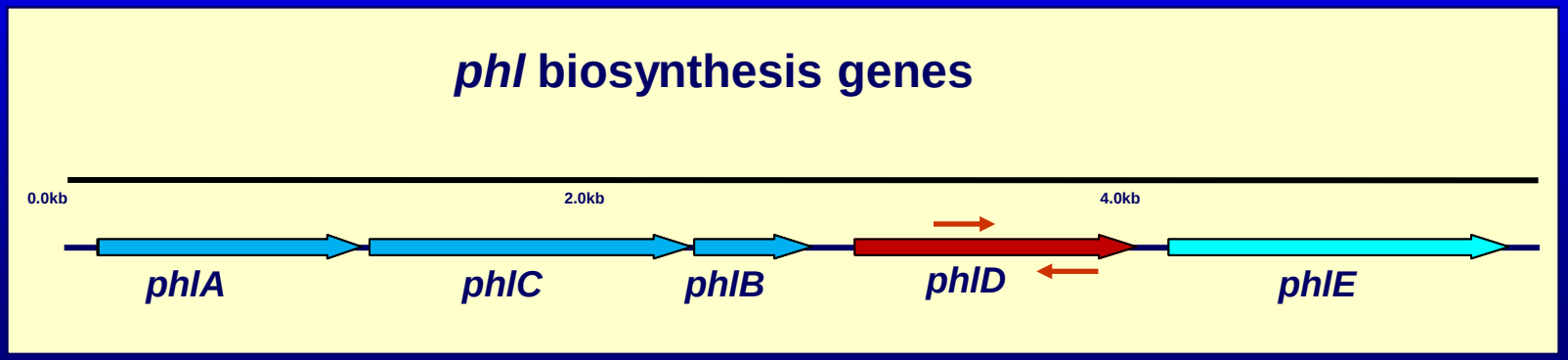
Genotype-specific *phlD* primers for real-time PCR

Genotype (strain)	Primers name	Primers sequence 5' to 3'	Positions	Tm [a] of primers
A (Pf-5)	A_Up	GCACGGTGGAGGTTGGC	507	66.7 C
	A_Low	GTGATCGTCACTTCCTGCAC	575	62.6 C
B (Q2-87)	B_Up	CACGCATCCCAATTGAG	477	59.4 C
	B_Low	CCGTTACCTCTTGCACC	573	58.0 C
D (Q8r1-96)	D_Up	AGTTGCAGGACCAGTTC	37	57.6 C
D (FTAD1r34)	D_Low	CATTAAAGATGTCGCACCG	148	61.9 C
I (FTAD1r36)	I_Up	GGTTCCAGGTCCAGTTG	26	56.8 C
	I_Low	CGTCAAGGACAGTGGCTTC	199	62.9 C

[a] Oligonucleotides were designed by Oligo 6.65 Primer Analysis Software and Tm of the primers were calculated by Oligo 6.65 using the nearest-neighbor thermodynamic method.



General scheme for enumeration of introduced and indigenous bacteria in the wheat rhizosphere by real-time PCR

10 ml of H_2O; vortex, sonicate

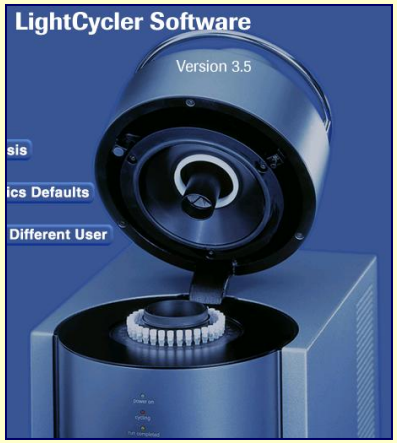
2 ml of root washes

UltraClean™ Soil DNA Isolation kit

Modified alternative protocol for wet soil samples

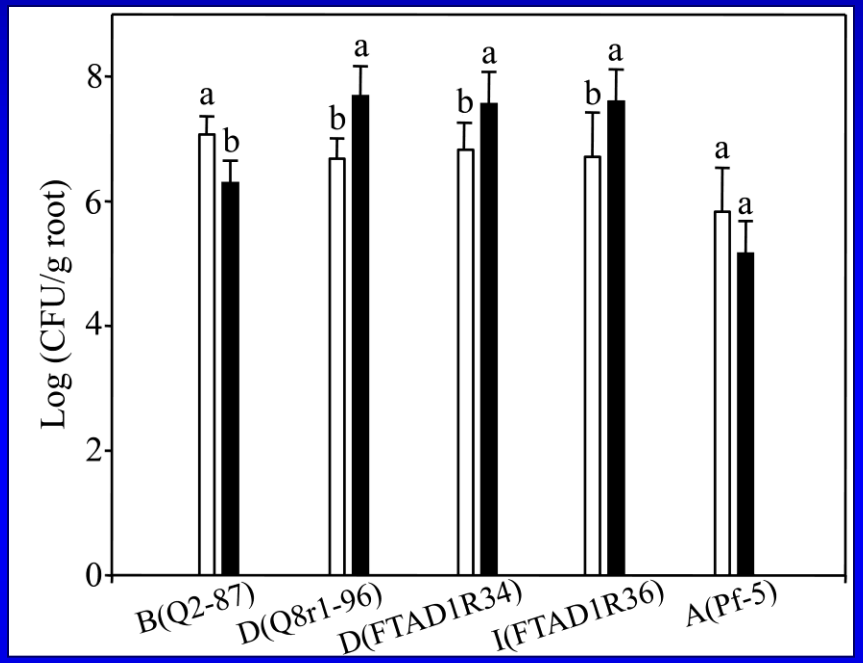
50 µL of DNA per sample

Real-time PCR



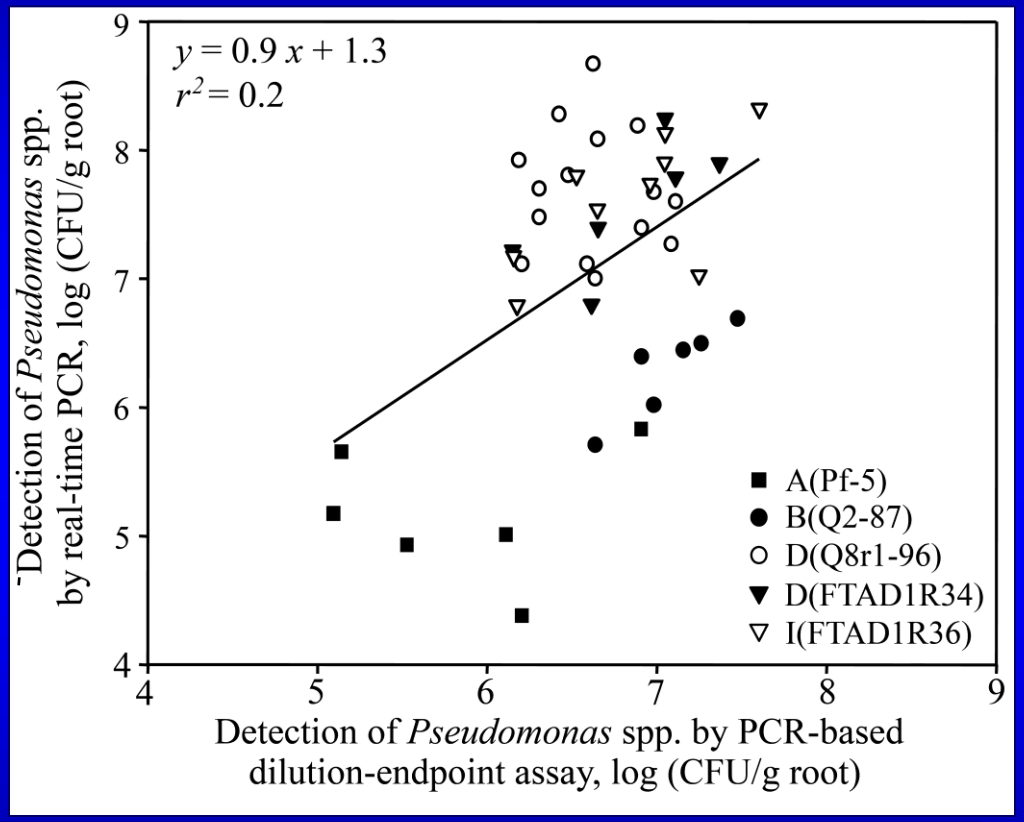
2 µL of DNA are used per PCR reaction

Comparison of population levels of DAPG-producing *Pseudomonas* spp. in the rhizosphere of wheat detected by real-time PCR and TD endpoint assay



Terminal dilution endpoint assay

real-time PCR



Regression analysis of population densities detected by real-time PCR and by a previously described TD endpoint assay indicated a significant linear relationship (P=0.0016, r^2=0.2). Each symbol represents the population of bacteria detected on roots plus adhering rhizosphere soil from a single seedling. **Mavrodi et al., 2007**

Comparison of real-time PCR and TD endpoint assay

Real-time PCR

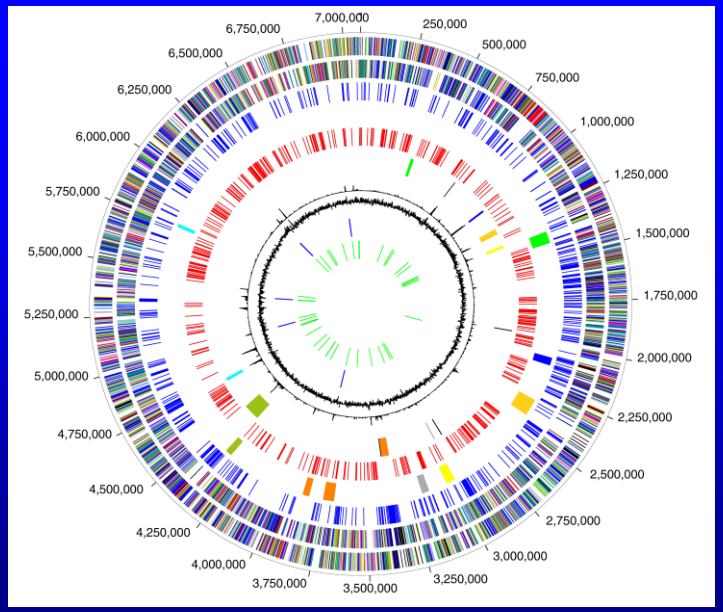
- Culture independent;
- Samples consist of purified DNA;
- Allows detection of dominant and subdominant genotypes;
- Allows detection of indigenous bacteria (i.e. those not tagged with antibiotic resistance);
- Detection limit on average is log 4 - 5 per rhizosphere;
- Cost per reaction ~ $4.60 (including cost of soil DNA extraction, and not including cost of equipment and service contract);
- Turnaround time is 2 days.

TD endpoint assay

- Culture dependent (inhibition may occur in mixed cultures during incubation step);
- Samples consist of frozen cell suspensions;
- Allows easy detection of only dominant genotypes;
- Works best for strains tagged with antibiotic resistance;
- Detection limit is log 3.3 CFU/g root;
- Cost per reaction ~ $3.50;
- Turnaround time is 5 days.

Thoughts for the Future

- Solving problems that are barriers to biocontrol reaching its full potential as an integral part of sustainable agriculture will require multi-disciplinary research focusing on the biocontrol process at all levels, from the genome to ecosystem scale.
- Developing tools to track the establishment and fate of BCAs will continue to be an important component of the multi-disciplinary research effort.




Thank you